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Evidence for PQQ as cofactor in 3,4-dihydroxyphenylalanine (dopa) decarboxylase of pig kidney

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Pig kidney 3,4-dihydroxyphenylalanine (dopa) decarboxylase (EC 4.1.1.28) was purified to homogeneity. Treatment of the enzyme with phenylhydrazine (PH) according to a procedure developed for analysis of quinoproteins gave products which were identified as the hydrazone of pyridoxal phosphate (PLP) and the C(5)-hydrazone of pyrroloquinoline quinone (PQQ). This method failed, however, in quantifying the amounts of cofactor. Direct hydrolysis of the enzyme by refluxing with hexanol and concentrated HCl led to detachment of PQQ from the protein in a quantity of 1 PQQ per enzyme molecule. In view of the reactivity of PQQ towards amines and amino acids, we postulate that it participates as a covalently bound cofactor in the catalytic cycle of the enzyme, in interplay with PLP. Since several other enzymes have been reported to show the atypical behaviour of dopa decarboxylase, it seems that the PLP-containing group of enzymes can be subdivided into pyridoxoproteins and pyridoxo-quinoproteins.

3,4-Dihydroxyphenylalanine decarboxylase; Pyrroloquinoline quinone; Pyridoxal phosphate; Cofactor; Quinoprotein; Hydrazone; (Pig kidney)

1. INTRODUCTION

During the past few years, we demonstrated that many metallo-oxidoreductases are in fact metalloquinoproteins: bovine serum amine oxidase [1], porcine kidney diamine oxidase [2], human placental lysyl oxidase [3], galactose oxidase (unpublished), dopamine β-hydroxylase [4], soybean lipoxygenase-1 (unpublished). The presence and quantity of PQQ could be established by using the hydrazine procedure [5], derivatizing the covalently bound PQQ to the stable hydrazone before protein hydrolysis was carried out. All the aforementioned enzymes have been studied for many years, some of them being considered to contain Cu ions and pyridoxal phosphate (PLP), others to contain only a metal ion. Meanwhile the quinoprotein nature has been confirmed by other researchers. Thus, the conclusion must be that the cofactor PQQ has been overlooked in these well-known enzymes, leading to the question of whether this might also apply to other well-established groups of enzymes.

Transaminases and amino acid decarboxylases have been studied for decennia and the presence or requirement of PLP well documented. Upon screening the literature, it appears, however, that another chromophore with an absorption maximum in the 330–340 nm range is mentioned in several cases: rat liver ornithine aminotransferase (EC 2.6.1.13) [6], ω-amino-acid : pyruvate aminotransferase (EC 2.6.1.18) from a *Pseudomonas* species [7], mammalian 4-aminobutyrate transaminase (EC 2.6.1.19) [8,9], dopa decarboxylase or aromatic-L-amino-acid decarboxylase (EC 4.1.1.28) from pig kidney [10] and from rat liver [11]. Low pH, high pH or dialysis against hydroxylamine-containing buffers removed the PLP from these enzymes, but not the unknown compound. Since the properties of the latter were strongly

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reminiscent of covalently bound PQQ, we attempted to prove this idea by applying the hydrazine method to a well-known representative of the aforementioned examples, namely pig kidney dopa decarboxylase.

2. MATERIALS AND METHODS

2.1. *Enzyme purification and assay*

Dopa decarboxylase was purified from 430 g fresh pig kidneys according to [10] with the following modifications: DEAE-Sepharose was used instead of DEAE-cellulose; the final gel filtration step on Sephadex G-100 was replaced by hydrophobic interaction chromatography. The latter was performed on a phenyl-Sepharose CL-4B column (2 × 6 cm), equilibrated with 0.4 M sodium phosphate buffer, pH 6.5. Application of the sample and washing of the column occurred with the same buffer. Elution was performed with a gradient ranging from 0.4 to 0.001 M sodium phosphate buffer, pH 6.5.

The enzyme was assayed by a known procedure converting the produced dopamine into a spectrophotometrically measurable compound, namely method A in [12].

2.2. *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis was performed on gradient gels (8–25%), using the Pharmacia Phastsystem equipment according to the instructions given by the manufacturer. Protein staining occurred with Phastgel blue.

2.3. *Derivatization with phenylhydrazine (PH)*

Derivatization of 2 ml enzyme solution (1.1 mg/ml in 10 mM sodium phosphate, pH 6.5) with PH occurred under the conditions required for hydrazone adduct formation [5]. Retention times and absorption spectra of adducts isolated from the enzyme, the model hydrazone of PQQ [5] and of PLP [13], were determined with HPLC and photodiode array detection [5].

2.4. *Extraction of PQQ by the hexanol procedure*

To a solution of 10 ml enzyme (0.55 mg/ml in 10 mM sodium phosphate buffer, pH 6.5), 10 ml concentrated HCl and 4 ml *n*-hexanol were added. The mixture was refluxed for 4 h. After cooling to room temperature, the organic layer was removed and washed with water until the washings were neutral. The fluid was then evaporated using a rotary film evaporator under reduced pressure. The remaining solids were dissolved in 550 μl methanol, the solution mixed with 300 μl of 10 mM sodium phosphate buffer, pH 6.5, and heated for 10 min at 55°C. Inspection for PQQ and its adducts occurred with HPLC and photodiode array detection [5]. The presence of free PQQ was also checked with a biological assay [14].

3. RESULTS AND DISCUSSION

3.1. *Purity of the enzyme*

The purification procedure yielded a final preparation which was homogeneous, as judged from polyacrylamide gel electrophoresis, showing only one band on protein staining. The band corresponded to M_r approx. 100 000, in agreement with the value of M_r 103 000 considered as the most reliable for pig kidney dopa decarboxylase [12]. The part of the absorption spectrum above 300 nm was similar to that reported by others [15], having an $A_{333\ nm}/A_{420\ nm}$ ratio of 2.70 and an $A_{280\ nm}/A_{333\ nm}$ ratio of 8.2 at pH 6.5 (unfortunately, spectra below 300 nm have not been published so that the latter ratio cannot be compared).

3.2. *Derivatization with PH*

In contrast to the results obtained with all quinoproteins investigated so far, application of the hydrazine method to dopa decarboxylase did not yield adduct on eluting the Seppak cartridge with methanol, but elution of coloured material required methanol containing 0.5% (v/v) concentrated ammonia. Investigation of this eluate with HPLC showed the presence of a multitude of coloured components and the presence of two small peaks, denoted A and B (fig.1). Absorption spectra of peaks A and B (corrected for the background) were identical to those of the C(5)-hydrazone of PQQ and PLP, respectively (fig.1). Also, the retention times were in complete agreement with those of the model compounds. The amounts of the hydrazones obtained from the enzyme were, however, far too low. This could either result from inadequate reaction of PH with the cofactors under the applied conditions and uncontrolled side reactions of PH, or from insufficient proteolysis of the derivatized enzyme. These points are currently under investigation. Thus, although the hydrazine method demonstrated the presence of PLP and PQQ in the enzyme, it was unsuitable for quantifying them (of course, PLP can be determined by a procedure under denaturing conditions [13]).

3.3. *The hexanol extraction procedure*

Recently we investigated galactose oxidase for the presence of PQQ. Since the main part of the enzyme preparation has the cofactor in a reduced form, the hydrazine method seemed unfeasible and a novel method, the so-called hexanol extraction procedure, was developed and applied successfully (to be published). Briefly, the rationale behind this procedure is the following: the higher aliphatic alcohol hexanol (the high boiling point creates the

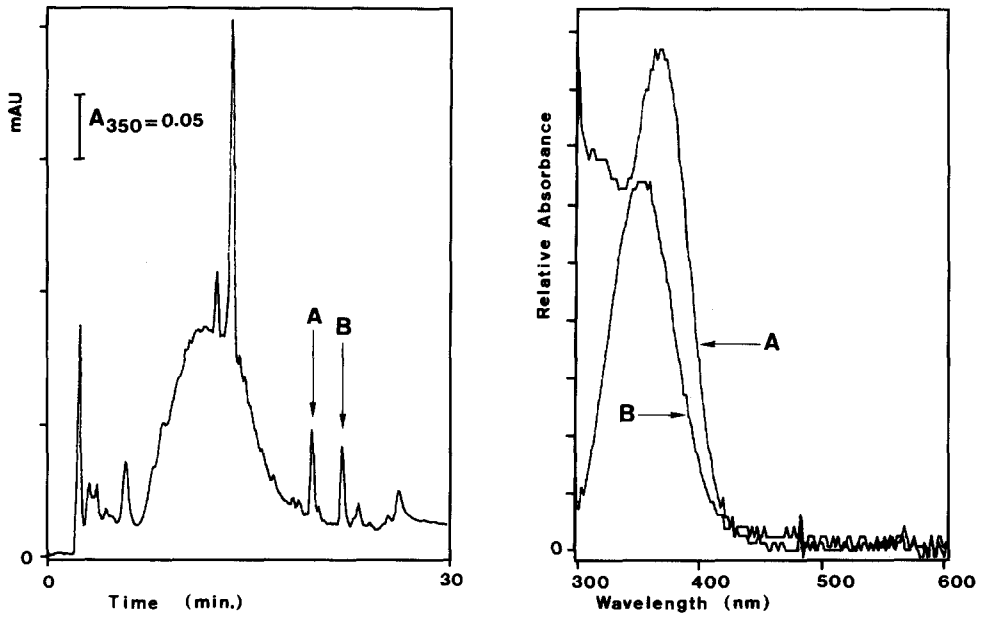

Fig.1. HPLC of the adducts isolated from PH-derivatized enzyme. The chromatogram (left) of the eluting species, monitored at 350 nm, and the absorption spectra of peaks A and B (taken with the photodiode array detector at the apex of the peaks and corrected for background) are shown (right).

high temperatures required for detachment of PQQ) forms an adduct with PQQ; the adduct becomes extracted into the organic solvent so that it escapes unwanted attack by other nucleophiles; the strong acid detaches PQQ or its adduct from the protein and protonates the carboxylic acid groups so that extraction becomes possible.

Application of the procedure to the enzyme gave a number of compounds, as revealed by the HPLC chromatogram (fig.2). Peak A appeared to be PQQ (most probably resulting from adduct decomposition during the last steps in the procedure), as judged from identical behaviour of authentic material with respect to retention time and absorption spectrum and activity in the biological assay. Peak B had properties identical to those of a model compound prepared by applying the hexanol procedure to PQQ. Although the other peaks were not further investigated, they are clearly derived from PQQ (e.g. see the absorption spectrum of peak C in fig.2), the characteristics of the spectra indicating that the conditions during enzyme hydrolysis probably lead to esterification or to diadduct (at the C(5) as well as C(4) position) formation of PQQ. PLP or PLP-like compounds were not observed in the chromatogram, suggesting that this cofactor is not converted into hexanol-soluble compounds. This was confirmed indeed by application of the hexanol procedure to authentic PLP.

3.4. *Quantification of PLP and PQQ*

The quantity of PLP in dopa decarboxylase has been reported to be 1 PLP per enzyme molecule [12,15]. From the absorption spectrum of the enzyme preparation in which this was determined [15], and using an M_r of 103 000, it was calculated that the molar absorption coefficient of the enzyme at 333 nm is 6200 $M^{-1} \cdot cm^{-1}$. The amount of PQQ in the enzyme was calculated from the areas of peaks in an HPLC chromatogram taken at 249 nm (the same peaks as shown in fig.2 for 350 nm detection), and transforming the sum of the areas into PQQ by using calibration values (this is allowed since differences in the absorption coefficients between PQQ and its adducts or esters are negligible at this wavelength). From this, a quantity of 1.03 PQQ per enzyme molecule was calculated. The conclusion is, therefore, that pig kidney dopa decarboxylase contains 1 PLP and 1 covelantly bound PQQ per enzyme molecule.

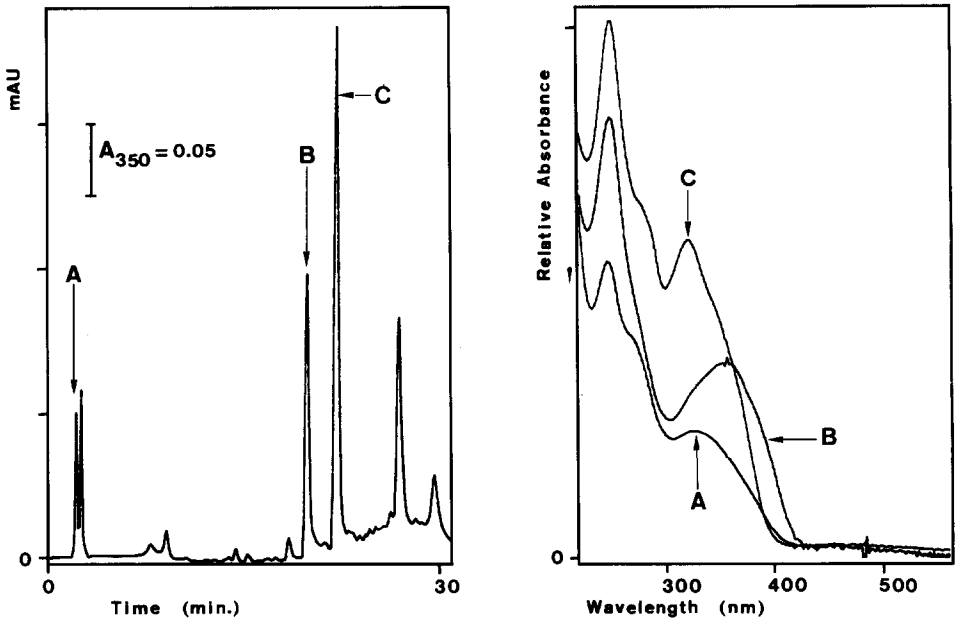

Fig.2. HPLC of products obtained with the hexanol extraction procedure. The chromatogram (left) was taken at 350 nm and the spectra of peaks A–C were taken with the photodiode array detector.

3.5. *Implications*

The conclusion that dopa decarboxylase contains PQQ makes it highly probable that this also applies to the enzymes with a similar unknown compound, mentioned in section 1. It seems, therefore, that PLP-containing enzymes can be subdivided into pyridoxoproteins and pyridoxo-quinoproteins.

Removal of PLP from dopa decarboxylase leads to enzyme inactivation and subsequent supplementation with PLP to restoration [10]. Although these observations show that PLP is necessary for activity, this should not be interpreted as demonstrating that PQQ has a passive role. PQQ forms adducts with amines and amino acids [16] and under certain conditions, the adducts are very rapidly converted in a cyclic process ([17] and unpublished). Since PLP has similar chemical activities, an interplay between the two cofactors in the catalytic cycle is an attractive supposition. Anyhow, it is clear that interpretations of spectroscopic and mechanistic data concerning the group of enzymes, indicated here as pyridoxo-quinoproteins, should be reconsidered as well as further research carried out before a sound proposition can be made.

The present finding is another illustration of the large versatility and the wide distribution of the cofactor PQQ. It is also illustrative for the involvement of PQQ in the conversion or biosynthesis of many mammalian bioregulators, in this case being the cofactor of dopa decarboxylase as well as dopamine β-hydroxylase, enzymes operating in the route of noradrenaline biosynthesis. Therefore, enzymological research on quinoproteins could provide important contributions to understanding physiological processes in mammals.

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